Research article

NKILA is a novel suppressor of local recurrence in women breast malignant phyllodes tumor patients via inhibition of the NF-κB pathway

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ABSTRACT

The aim of the present study was to explore the functional mechanism of NF-Kappa B-interacting Long non-protein coding RNA (NKILA) in breast malignant phyllodes tumors (BMPTs). The expression and functional role of NKILA were investigated by performing qRT–PCR, Transwell assays, and CCK–8 assays in primary BMPT cells. A Kaplan–Meier curve was used to assess overall survival (OS) and local recurrence-free survival (LRFS). The location and expression levels of NKILA and P65 were determined by fluorescence in situ hybridization (FISH) and immunofluorescence (IF), respectively. NKILA was downregulated in patients with BMPT, especially in patients with local recurrence. NKILA had an antitumor effect and promoted the chemosensitivity of cells to cisplatin by blocking P65 phosphorylation and nuclear translocation. In conclusion, NKILA may be a potential therapeutic target for BMPT, especially for BMPT patients with local recurrence.

1. Introduction

Breast phyllodes tumor (BPT) is a rare fibroepithelial tumor accounting for up to 1 % of all breast tumors [1]. Based on the characteristics of cellular atypia, the mitotic activity of stromal cells, stromal overgrowth, and the tumor border, BPTs are classified as benign, borderline or malignant [2]. Breast malignant phyllodes tumors (BMPTs) account for 20 % of all BPTs; unlike benign and borderline phyllodes tumors (PTs), BMPTs have higher rates of local recurrence and distant metastasis [3,4]. Patients with BMPTs often have a long history of clinical treatment, and their symptoms are complicated by rapid growth of the tumor mass [5]. For patients with benign, borderline, and malignant BPTs, surgical mastectomy is the first treatment option, but the existing adjuvant chemotherapy and radiotherapy options for patients with BMPTs are uncertain. Moreover, it is currently highly difficult to cure patients with

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local recurrence and/or distant metastasis [6]. However, basic and clinical research on BMPTs has been limited due to small sample sizes, and the prognosis and clinical treatment of BMPT patients are difficult to predict due to the scarcity of comprehensive basic and clinical research [7]. Therefore, it is crucial to identify new biomarkers to predict outcomes and improve treatment efficacy for BMPT patients.

Long noncoding RNAs (lncRNAs) are a class of nonprotein-coding RNAs that are longer than 200 nucleotides [8], and they participate in various physiological and pathological processes involved in human evolution and disease [9,10]. We have previously reported that NF-κB-interacting lncRNAs are transcribed in the cytoplasm by the NF-κB transcription factor and bind to NF-κB/inhibitory protein κB (IκB), thus blocking IκB phosphorylation and NF-κB pathway activity, with the negative feedback between NKILA and NF-κB/IκB being fairly stable [11]. Later studies have shown that NKILA is involved in tumor immunity by sensitizing T cells to activation-induced cell death and promoting tumor immune evasion in breast cancer [9]. In other tumor types, NKILA reduces the oral squamous cell carcinoma malignant progress via the NF-κB pathway [12]. In colorectal cancer, NKILA serves as a biomarker for the early diagnosis and outcome of patients [13]. In gliomas, NKILA enhances the Warburg effect and angiogenesis, suggesting that it may be a potential therapeutic target [14]. In hepatocellular carcinoma, NKILA is a prognostic indicator of inhibited tumor metastasis by reducing epithelial–mesenchymal transition (EMT) through the regulation of the NF-κB/Slug pathway [15]. However, the role of NKILA in the occurrence and development of BMPT remains unknown.

In the present study, NKILA was downregulated in primary BMPTs compared to peri-normal tissue, and NKILA downregulation was strongly correlated with poor OS. Exploration of the relationship between NKILA and local recurrence of BMPT indicated that the expression of NKILA decreased as the local recurrence of BMPT increased, and NKILA downregulation was closely correlated with poorer local LRFS. The expression of NKILA was also verified in BMPT primary cells, including peri-normal, primary tumor, and recurrent tumor cells. With respect to gene biological function, NKILA inhibited the migration, invasion and proliferation abilities of primary recurrent BMPT cells. Moreover, NKILA increased the chemosensitivity of primary recurrent BMPT cells to cisplatin. Together, these results indicated that NKILA may be an effective prognostic factor and a potential therapeutic target against local BMPT recurrence and metastasis.

2. Methods and materials

2.1. Breast phyllodes tumor tissue samples

In total, 76 pairs of breast phyllodes tumor tissue samples, including normal and tumor tissue samples, were collected and kept at −80 °C in the Department of Breast Surgery, Zhujiang Hospital Southern Medical University (Guangzhou, China) from 2015 to 2022. The median survival time of the 76 patients with breast PTs was 63 months. Of the 76 patients, 6 had local skin recurrence. Primary cells from two patients, including cells from normal tissue, primary tumor tissue, and recurrent local skin tumor tissue, were successfully cultured to detect NKILA expression in different stages of breast phyllode tumor samples. The collection of patient samples was approved by the Research Scientific Ethics Committee of Zhujiang Hospital, Southern Medical University (Guangzhou, China).

2.2. RNA extraction and qRT–PCR detection of NKILA expression

Approximately 10 mg of breast phyllode tumor tissues and normal tissues were collected and placed in a clean mortar. Then, liquid nitrogen was added to the mortar, and the tissues were ground into powder. The resulting tissue powder and 1 ml of TRIzol were added to a 1.5 ml EP tube. Total RNA was extracted using liquid chromatography, and the RNA was reverse transcribed into cDNA by using a reverse transcription kit (Vazyme, China). cDNA was used to detect NKIL expression via PCR. The primers for NKILA were designed and synthesized by Generay (Shanghai, China). The PCR system was constructed according to the manufacturer's instructions and included SYBR Green Master Mix (Vazyme, China), primer sequences, and RNase-free water. The NKILA expression levels were calculated by the $2^{-\triangle\triangle Ct}$ method. $2^{-\triangle\triangle Ct}$ is a standardized calculation method of real-time fluorescence quantitative PCR (qRT-PCR), Ct is the cyclic threshold of gene amplification, $\triangle Ct$ = value of NKILA Ct - value of GAPDH Ct, $\triangle\triangle Ct$ = $\triangle Ct$ value of NKILA - $\triangle Ct$ value of reference gene, $2^{-\triangle\triangle Ct}$ = power (2, -$\triangle\triangle Ct$).

2.3. Primary cell extraction and culture

Fresh breast phyllode tumor tissues, including normal tissues, tumor tissues and local skin recurrence tissues, were immediately collected from the operating room at Zhujiang Hospital. The tissues were chopped and digested with collagenase types I and III (Sigma, USA) with shaking at 37 °C for 2 h in DMEM supplemented with 100 μg/ml streptomycin and penicillin. The samples were then filtered through strainers with different apertures. Primary cells were obtained by centrifugation at 4 °C and 1800 rpm (RCF: 650g) for 10 min and then cultured in F12 medium supplemented with 20 % fetal bovine serum (Thermo Fisher, Australia). During the culture period, the nonadherent cells were removed with fresh medium. Third- or fourth-passage breast phyllodes tumor primary cells were used to conduct molecular experiments.

2.4. Northern blot

This assay was used to detect the expression of NKILA in different breast phyllode tumor primary cells. Total RNA that was extracted from breast tumor primary cells was subjected to Northern blot assays as per the protocol provided by Roche (Basel,

Switzerland), where we used digoxin-labeled NKILA probes (50–100 μM) to hybridize overnight at room temperature (RT). Probe detection was performed using a digoxin Luminescent Detection Kit for Nucleic Acids (Roche, Basle, Switzerland).

2.5. Fluorescence in situ hybridization (FISH)

A FISH kit (Ribo^{TM}, Guangzhou) was used to detect NKILA expression in primary breast PT cells. In brief, 4×10^4 prepared cells were seeded onto cell sheet glass in 24-well plates, washed three times with phosphate-buffered saline (PBS) (Biosharp, China), fixed with 4 % paraformaldehyde (Biosharp, China) at RT for 10 min, and incubated with 200 μl of prehybridization buffer at 37 °C for 30 min. Then, 5 μM NKILA probe for FISH was added to the 24-well plates and incubated at 37 °C overnight in the dark. The hybridization buffer was used to wash the cells three times at 42 °C. The cell nuclei were stained with DAPI solution. Finally, images were acquired by laser confocal microscopy (Leica Microsystems). The results are representative of three independent experiments performed in triplicate.

2.6. Plasmid transfection

For NKILA plasmid transfection, cells were plated at 60–70 % per well in 6-well plates and transfected with specific plasmids against NKILA duplexes using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. The third- or fourth-passage BMPT primary cells were used to up-regulate NKILA by plasmids transfection, the efficiency of plasmids transfection was up to 75–80 %, and the transfected cells were immediately used to perform next functional experiments.

2.7. Transwell assay

Transwell assays were used to evaluate the migration and invasion abilities of primary breast PT cells after transfection with the NKILA plasmid. Transwell chambers were used to simulate the cell barrier. Unlike in the migration assay, a layer of Matrigel (BD Biosciences) (approximately 100 μl) was placed at the bottom of the Transwell chamber in the invasion assay. After stable transfection of the NKILA plasmid, 2,000 cells were seeded into the upper chambers in DMEM supplemented with 5 % FBS, and DMEM supplemented with 10 % FBS was added to the lower chambers. After approximately 8 h, the chambers were collected and quantified by imaged three random fields of view.

2.8. Cell Counting Kit-8 (CCK-8) assay

A CCK-8 assay was used to evaluate the proliferation ability of primary breast phyllodes tumor cells. Briefly, cells with or without stable transfection of NKILA were cultured in 96-well plates (2,000 cells/well). CCK-8 solution was added after 0, 12, 24, 36, 48, 60, and 72 h, and the cells were cultured for an additional 2 h. The absorbance at 450 nm was detected to assess the cell viability by a microplate reader (BioTek, USA). Three independent experiments were conducted in triplicate.

2.9. If

Immunofluorescence staining of P65 was performed to evaluate the effect of NKILA on the NF-κB signaling pathway in primary breast phyllodes tumor cells. After NKILA was overexpressed, the cells were fixed in tissue fixative solution for 15 min, permeabilized for 10 min with 0.1 % Triton X-100, blocked for 30 min with 1 % BSA at RT, and then incubated with a primary antibody against P65 (3033) for 2 h at RT. The cells were then incubated with secondary antibody for 1 h at RT, and the nuclei were stained using DAPI solution. Finally, images were acquired by laser confocal microscopy (Leica Microsystems). P65 expression in each group was statistically analyzed in five randomly selected fields of view (approximately 300 cells).

2.10. RNA sequencing (RNA-seq)

To explore the molecular mechanism of NKILA in the metastasis and recurrence of BMPT, we established BMPT primary cells overexpressing NKILA by transiently transfecting NKILA plasmid. Approximately, 2×10^7 cells were lysed and extracted by RNA extractor kit, the totally RNA was detected by library construction and sequencing at Guangzhou Gene Denovo Biotechnology Corporation.

2.11. Statistical analysis

All the statistical analyses were performed by Student's t-test by using GraphPad Prism 5.0, and the Kaplan−Meier analyses were conducted by nonlinear regression in GraphPad Prism 5.0. The results were obtained from three independent experiments, and the statistical analysis results are presented as the mean ± SEM.

3. Results

3.1. Low expression of NKILA indicates poor prognosis in BMPT patients

To explore the expression of NKILA in BPMT, we detected the NKILA RNA levels in the primary normal (N) and tumor (T) cells by Northern blot, and found that the NKILA expression was lower in primary BPMT tumor cells than in adjacent normal cells (Fig. 1A).

NKILA expression was analyzed in 76 pairs of normal and primary tumor tissue samples from BMPT patients from Zhujiang Hospital by qRT–PCR. NKILA expression was significantly lower in primary BMPT tissue samples than in adjacent normal tissue samples (Fig. 1B). Based on NKILA expression, the 76 patients in the BMPT cohort were divided into two groups, namely, the NKILA high-expression group (n = 29) and the NKILA low-expression group (n = 47). K–M curves revealed that patients with lower NKILA expression had poorer OS and LRFS (Fig. 1C and D). The correlation between NKILA expression and the clinicopathological characteristics of 76 BMPT patients was analyzed. Lower NKILA expression was strongly related to tumor size, histological grade, recurrence, metastasis, and Ki-67 level, but it was not associated with age or menopausal status (Table 1). These results indicated that aberrant NKILA expression may be a prognostic marker for malignant progression in BMPT patients.

3.2. NKILA expression decreases with BMPT recurrence

BMPT is associated with a high recurrence rate, and the degree of malignancy is positively correlated with the number of local recurrences of BMPT [16]. BMPT tissue samples, including adjacent normal tissues, primary tumor tissues, and local recurrent tumor tissues, from six patients were used to detect NKILA expression by qRT–PCR. The primers used for NKILA are shown in Table 2. NKILA expression was lowest in the local recurrent tumor samples from the six BMPT patients (Fig. 2A). NKILA expression was also detected in primary BMPT cells, including adjacent normal cells, primary tumor cells, and local recurrent tumor cells, from two patients by FISH. NKILA expression was lowest in the local recurrent tumor cells among the other cells from the two BMPT patients (Fig. 2B). These results suggested that NKILA expression was lower in local BMPT recurrence samples than in primary tumor and normal samples.

3.3. NKILA inhibits migration, invasion and proliferation but increases the chemosensitivity to cisplatin of primary BPMT recurrent cells

To explore the functional role of NKILA in primary BMPT recurrent cells, an NKILA overexpression plasmid was transiently transfected into primary BMPT recurrent cells from two patients. The clinicopathological characteristics of the 2 patients were showed in Supplemental Table 1. The overexpression efficiency was up to 100 times that of the vector plasmid (Fig. 3A). Transwell assays
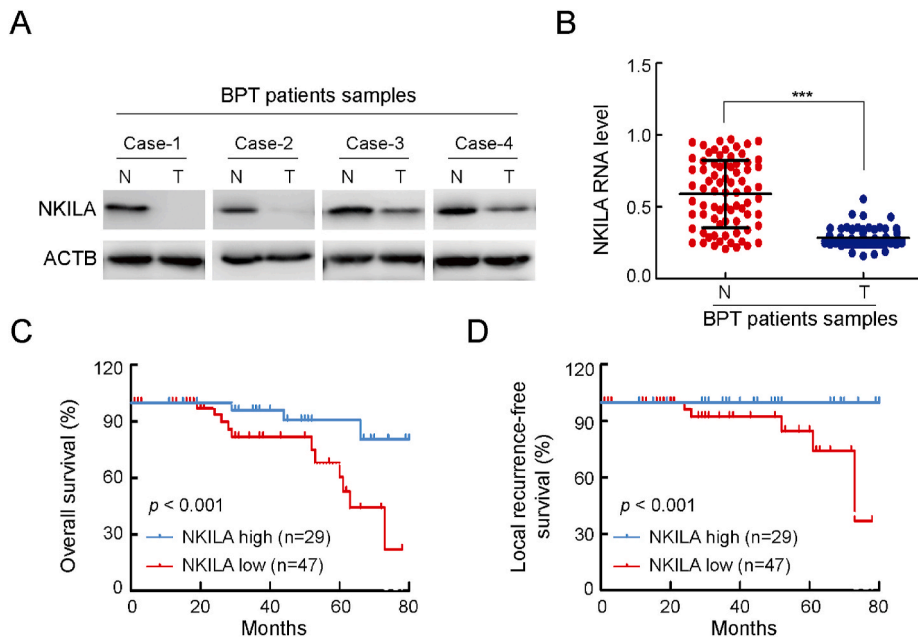

Fig. 1. Influence of lncRNA NKILA on the prognosis of BMPT patients. **A** The expression of NKILA in primary BMPT cells and normal cells was detected by northern blot. **B** The expression of NKILA in BMPT tissues and normal tissues was measured by qRT–PCR (n = 76, ***$p < 0.001$). **C** Based on the NKILA expression in 76 pairs of BMPT patients, patients with high NKILA expression (n = 29) had better OS than patients with low NKILA expression (n = 47) according to Kaplan–Meier curves (***$p < 0.001$ by paired Student's tests, mean ± SEM). **D** Kaplan–Meier curves showing that patients with high NKILA expression had better RFS than patients with low NKILA expression (***$p < 0.001$ by paired Student's tests, mean ± SEM).

Table 1
Correlation of NKILA expression with the clinicopathologic status of 76 breast malignant phyllodes tumor patients.

Characteristics	n	NKILA expression		P
		High	Low	
Total	76	29	47	
Age (years)				1.000
≤40	25	10	15	
>40	51	19	32	
Menopausal status				1.000
Premenopausal	32	12	20	
Postmenopausal	44	17	27	
Tumor size (cm)				0.007
≤2.5	29	17	12	
>2.5	47	12	35	
Histological grade				0.002
Grade I/II	34	20	14	
Grade III	42	9	33	
Recurrence				0.033
Yes	39	10	29	
No	37	19	18	
Metastasis				0.019
Yes	37	9	28	
No	39	20	19	
Ki-67 level				0.033
≤15 %	37	19	18	
>15 %	39	10	29	

Table 2
Primer sequences of NKILA for quantitative real-time PCR.

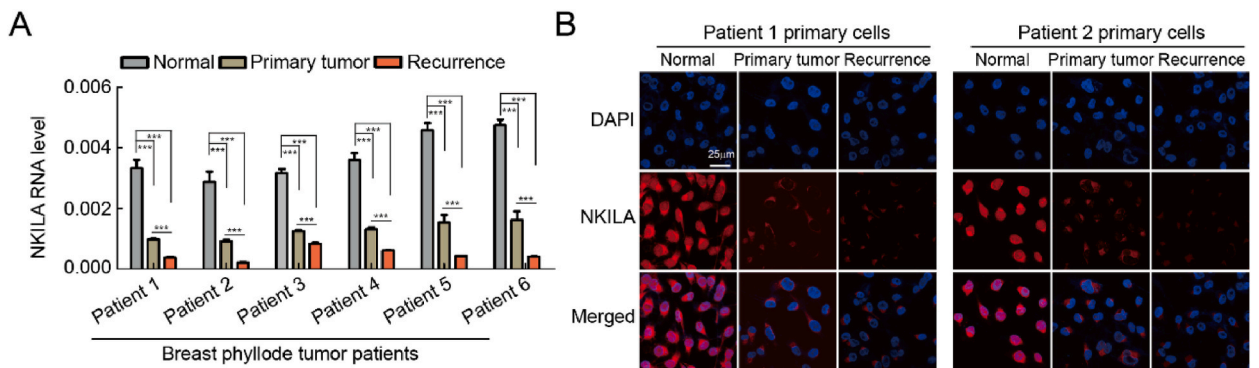| Characteristics	Forward Primer (5′to 3′)	Reverse Primer (5′to 3′)
NKILA	AACCAAACCTACCCACAACG	ACCACTAAGTCAATCCCAGGTG
GAPDH	ATCACCATCTTCCAGGAGCGA	CCTTCTCCATGGTGGTGAAGAC

Fig. 2. NKILA expression is associated with the development of BMPT. **A** The expression of NKILA in primary cells from six BMPT patients, including normal, primary tumor, and recurrent tumor cells, was measured by qRT−PCR (***$p < 0.001$). **B** The expression of NKILA in primary BMPT cells, including normal, primary tumor, and recurrent tumor cells, from two patients was detected by FISH (***$p < 0.001$).

showed that NKILA overexpression significantly inhibited the migration and invasion abilities of the primary BMPT recurrent cells from two patients (Fig. 3B). A CCK-8 assay showed that NKILA overexpression significantly inhibited the proliferation of primary BMPT recurrent cells from two patients (Fig. 3C). These data indicated that NKILA plays an important molecular role in the progression of BMPT recurrence. At present, there is no effective adjuvant therapy, including chemotherapy and radiotherapy, for BMPT, especially for patients who experience BMPT recurrence [17]. The present study assessed whether NKILA overexpression enhances the sensitivity of primary BMPT recurrent cells to cisplatin. The IC50 of cisplatin in primary BMPT cells from two patients was significantly reduced after NKILA overexpression (Fig. 3D). These data revealed that NKILA may be a supplementary target for improving the cisplatin chemosensitivity of BMPT patients who experience recurrence.
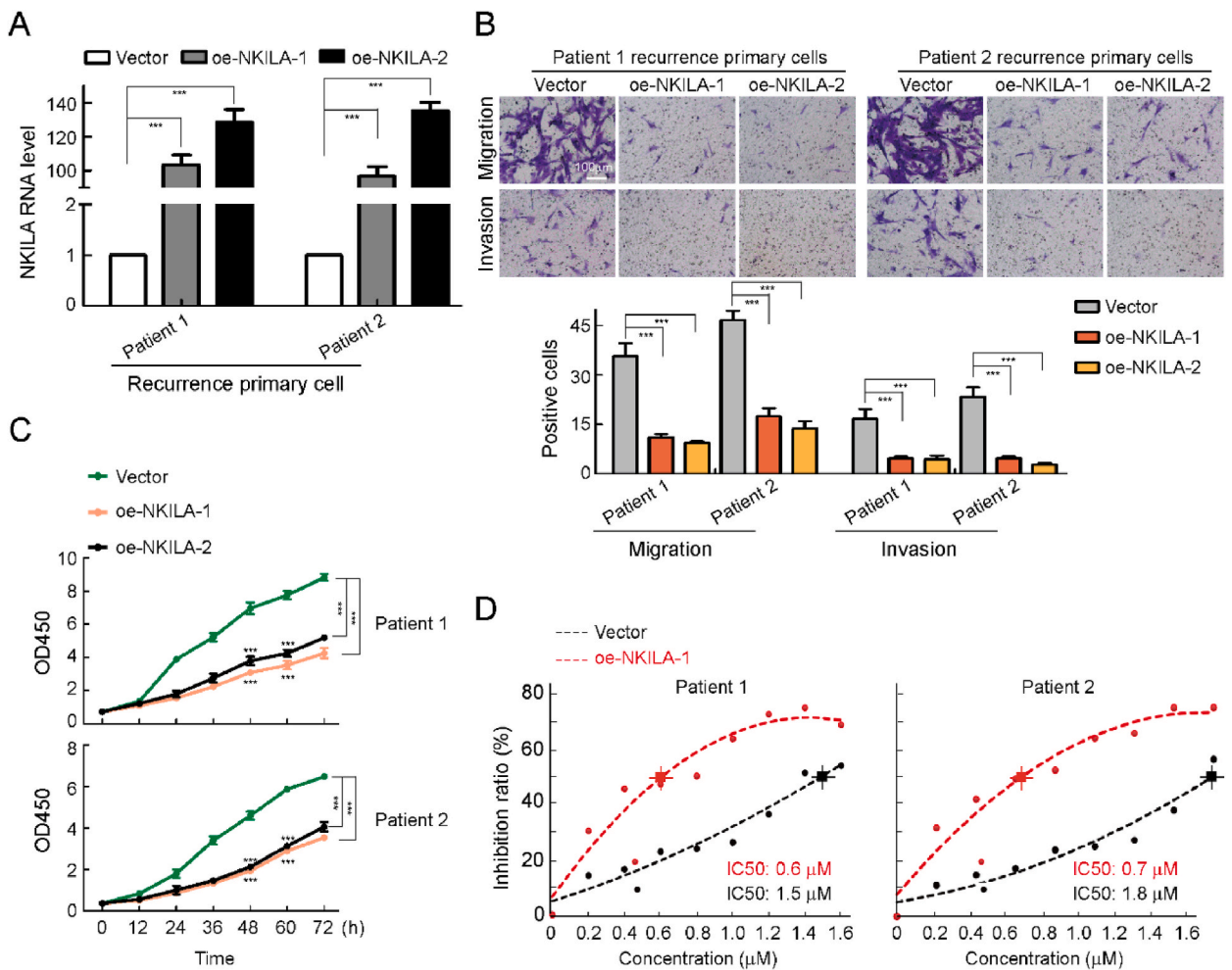
Fig. 3. NKILA inhibits the malignant biological ability of primary recurrent BMPT cells and promotes chemosensitivity to cisplatin. **A** In primary recurrent BMPT cells from two patients, the overexpression efficiency of NKILA was measured by qRT–PCR (***$p < 0.001$). **B** Representative images showing that NKILA inhibited the migration and invasion of primary recurrent BMPT cells from two patients (scale bar = 100 μm) according to the Transwell assay results (***$p < 0.001$ by paired Student's test, mean ± SEM). **C** The influence of NKILA on the proliferation of primary recurrent BMPT cells from two patients was determined using a CCK-8 assay at different time points (***$p < 0.001$ by paired Student's test, mean ± SEM). **D** The influence of NKILA on the chemosensitivity of primary recurrent BMPT cells from two patients to cisplatin was determined by the CCK-8 assay (***$p < 0.001$ by paired Student's test, mean ± SEM, oe means overexpression).

3.4. NKILA suppresses the NF-Kappa B signaling pathway by inhibiting P65 nuclear translocation in primary recurrent BPMT cells

To explore the possible molecular mechanism of NKILA in BMPT recurrence in primary cells, the effect of NKILA on the NF-Kappa B signaling pathway was investigated. RNA-seq was performed in cells with increased NKILA expression, and GSE signaling pathway analysis indicated that overexpression of NKILA impaired the NF-κB signaling pathway (Fig. 4A). Previous studies indicated that NKILA inhibited NF-κB signaling pathway in breast cancer, the biological process of Inflammation cytokines, such as TNF-α induced p65 nuclear translocation was a representative process of NF-κB signaling pathway activation. In this study, the expression and location of p65 were detected after NKILA overexpression. The expression of p65 in the nucleus decreased with increasing NKILA overexpression, as detected by IF, WB, FISH and qRT–PCR (Fig. 4B and C). These results indicate that NKILA impairs the NF-Kappa B pathway by inhibiting P65 phosphorylation and subsequent nuclear translocation.

4. Discussion

Local recurrence and distant metastasis are important processes that predict the prognosis of BMPT patients [18]. There is a tendency toward further deterioration of pathological characteristics after local recurrence and distant metastasis, and the mean patient survival time is only 5 months, with 88 % of patients not surviving [19,20]. To date, the molecular mechanism underlying the recurrence and metastasis of BMPT is largely unknown. Consequently, there is an urgent need for therapeutic strategies to prevent
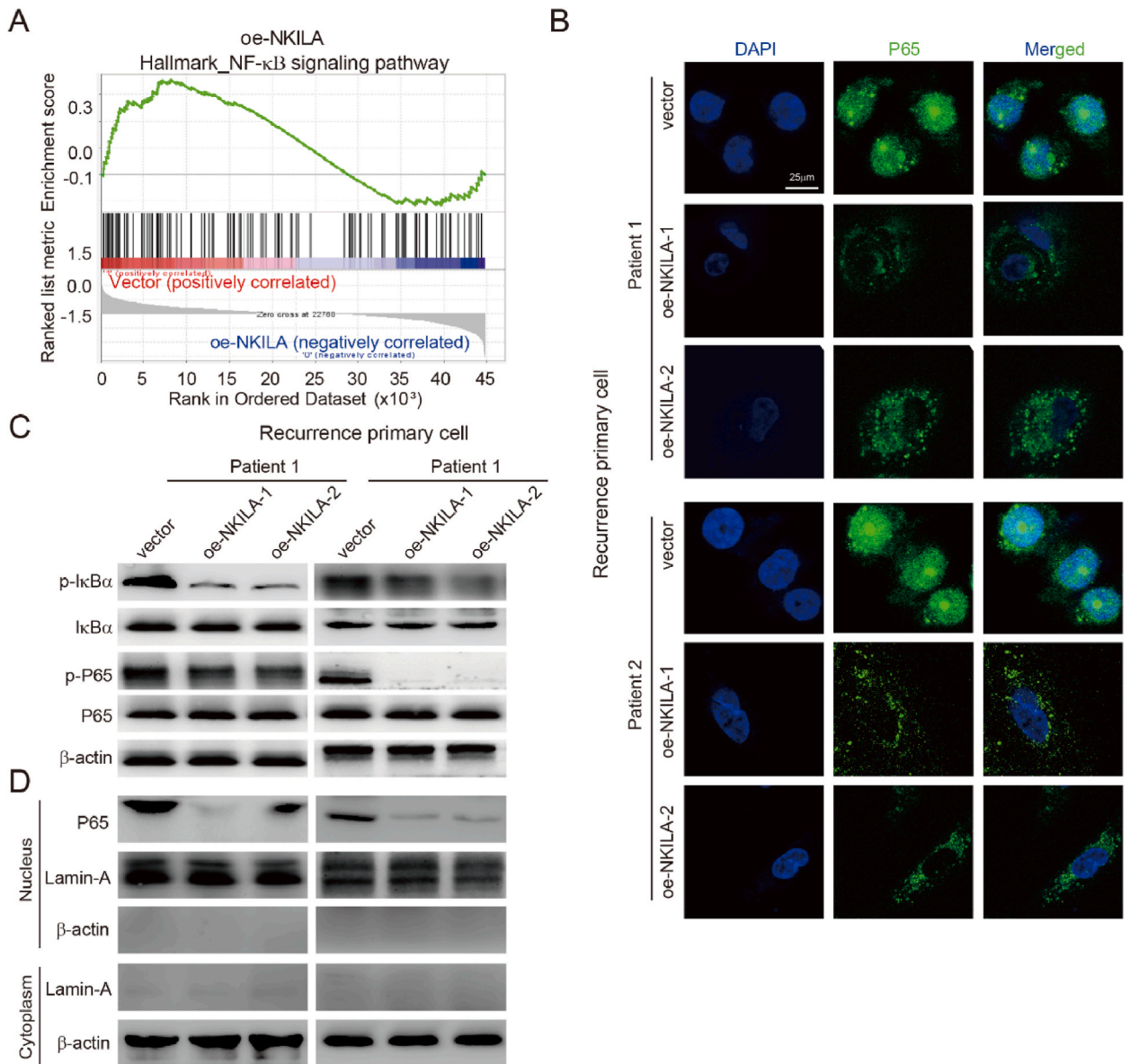
Fig. 4. NKILA inhibits the malignant biological ability of primary recurrent BMPT **cells by suppressing the NF-Kappa B signaling pathway. A** GSE signaling pathway analysis showed that the overexpression of NKILA impaired the NF-Kappa B signaling pathway. **B** The expression level and location of NKILA in cells overexpressing NKILA were detected by immunofluorescence. **C** Total and phosphorylated IκBα and P65 were detected by western blot analysis. **D** The expression of P65 in the cytoplasm and nucleus was detected by western blot analysis. (oe means overexpression, Lamin-A and β-actin were the loading controls of nucleus and cytoplasm).

local recurrence and distant metastasis in BMPT patients.

Many studies have indicated that abnormal and dysfunctional lncRNAs play crucial roles in the occurrence and malignant development of tumors, such as breast cancer [21], colon cancer [22], lung cancer [23], glioma [24], and liver cancer [25]. We have previously reported that NKILA is involved in the malignant progression of breast cancer, including metastasis [10] and tumor immune invasion [9]. The present study explored the relationships of NKILA with malignant progression and the chemotherapeutic effect in BMPT. NKILA was aberrantly downregulated in BMPT tissues with or without local recurrence compared to normal tissues, and aberrant downregulation of NKILA was correlated with poorer OS and LRFS. NKILA overexpression in primary recurrent BMPT cells significantly inhibited migration, invasion and proliferation, as well as promoted chemosensitivity to cisplatin.

In the present study, the expression and molecular function of NKILA were investigated in BMPT tissues and primary cells. NKILA was downregulated in the primary tumor and local recurrence tissues and primary cells of BMPT patients compared to the adjacent normal tissues and primary cells of BMPT patients, especially in the primary local recurrent BMPT cells. Moreover, the present study

demonstrated that NKILA expression decreased with local BMPT recurrence, which suggested that NKILA expression is associated with BMPT malignancy because the histopathology grade is worse for patients with local BMPT recurrence and metastasis.

Increased expression of NKILA has been shown to inhibit the proliferation, migration and metastasis of tumor cells, including breast cancer cells [10], non-Hodgkiń's lymphoma cells [26], and colon adenocarcinoma cells [27]. Similarly, the present study demonstrated that increased expression of NKILA inhibited the migration, invasion and proliferation of primary recurrent BMPT cells, which suggested that NKILA suppresses the malignant behaviors of BMPT cells. At present, the therapeutic effects of chemotherapy and radiotherapy are unsatisfactory for patients with BMPT, especially those with local recurrence or distant metastasis, and there is a lack of effective treatment strategies [18]. The present study investigated whether NKILA can serve as a therapeutic target to enhance chemosensitivity in patients with BMPT. Increased expression of NKILA enhanced the chemosensitivity to cisplatin of primary recurrent BMPT cells. Together, the present data suggest that NKILA, a tumor suppressor gene, may be a key prognostic marker in BMPT and a molecular target for improving the chemosensitivity of BMPTs to chemotherapy drugs.

We previously reported that NKILA directly binds to the functional domains of IκB, which is a signaling protein of the NF-κB pathway, thereby serving as an NF-κB regulator to suppress breast cancer [10]. The present study verified that NKILA may play a suppressive role by inhibiting the NF-κB pathway. Future studies will explore the mechanism relationship between NKILA and the NF-κB pathway in BMPT in vitro and in vivo.

5. Limitation

The major limitations of the present study are that the function and mechanism significance of NKILA low expression in BMPT have not been verified in vivo. Future research will be undertaken to explore the function and mechanism significance of NKILA low expression in BMPT by using different models. In addition, 76 BPMT cohort was used to detect the correlation of NKILA expression with clinicopathologic status of 76 BMPT patients, the sample size is too small, this study might have limited generalizability, we will increase the sample size by contacting other hospital.

Data availability

The present data can be obtained from the corresponding author upon reasonable request.

Declarations

Financial & competing interests disclosure The authors have no relevant financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. No writing assistance was utilized in the production of this manuscript.

CRediT authorship contribution statement

Ying Mi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Le Yan:** Resources, Data curation. **Haiyun Jin:** Resources, Investigation. **Ming Jin:** Resources. **Di Zhu:** Resources. **Hongyan Huang:** Software, Resources. **Kai Han:** Methodology, Data curation. **Jibo Huang:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors have no relevant financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. No writing assistance was utilized in the production of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33259.

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